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Frequent S100A4 Expression with Unique Splicing Pattern in Gastric Cancers: A Hypomethylation Event Paralleled with E-cadherin Reduction and Wnt Activation[1]

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Abstract

S100A4 promotes cancer metastasis, but its overall status and splicing manner during gastrocarcinogenesis remains less known. We therefore examined S100A4 frequencies, splicing pattern(s) and the underlying reason(s) for S100A4 expression in gastric cancers. Immunohistochemistry revealed frequent S100A4 expression in intestinal gastric cancers (37/45; 82%) and diffuse gastric cancers (12/20; 60%), but uncommon in noncancerous epithelia (0/12), chronic gastritis (2/24; 8%), and intestinal metaplasia (3/15; 20%). Of 65 primary tumors, 18 were found with focal S100A4 expression, while their LN metastases showed homogenous distribution. S100A4-oriented reverse transcription–polymerase chain reaction yielded a transcript containing exons 1, 3, and 4 (AS1) in 20% of noncancerous, 84% premalignant, and 92% tumor tissues and a transcript harboring exons 1 to 4 (AS2) in 65% of gastric cancers (GCs), 26% premalignant but none in noncancerous tissues. Further analyses found AS1 expression in stromal but not epithelial cells of premalignant tissues, absence of AS2 in endoscopic inflammatory mucosa, and the coexistence of AS1/AS2 in the cultured fibroblasts. Methylation DNA sequencing revealed hypermethylation of four critical CpG sites within S100A4 intron first among S100A4-negative gastric tissues and hypomethylation in S100A4-expressing GC tissues/cell lines. E-cadherin reduction and Wnt activation were common in gastric cancers, which were closely correlated but unnecessarily overlapped with S100A4 expression. Our findings suggest that S100A4 expression is closely related with GC formation, which, as a hypomethylation event, is accompanied with E-cadherin reduction and Wnt activation. The preferential S100A4 AS2 expression in GC cells would have potential values in GC surveillance and prognostic assessment.

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Introduction

Gastric cancer (GC) is one of the commonest malignancies in the world and the major cause of cancer-related deaths in China [1]. The prognosis of GCs remains poor owing to its later diagnosis and strong tendency of local invasiveness and distal metastasis [2]. Therefore, exploration of genetic alterations closely linked with GC formation and dissemination would be of great value in prevention, early detection, and prognostic evaluation of this malignancy.

S100A4 protein is the well-studied member of S100 family [3]. It is also named as metastasis-associated Mts1 and fibroblast-specific protein 1 (FSP1) because of its close association with cancer metastasis and constitutive expression in fibroblasts [4,5]. Moreover, S100A4 exerts multiple biological effects on proliferation and apoptosis, cell motility and adhesion, extracellular matrix remodeling, and angiogenesis of cancer cells [6]. For example, transfection of S100A4 could enhance the metastatic potential of bladder and mammary carcinoma cells [7,8], whereas blockage of its expression could reduce cancer

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metastatic capacity [9]. Although the tumor-promoting effects of S100A4 have been documented in a variety of cancers, no report has been so far available concerning the pattern(s) of S100A4 expression during stepwise gastrocarcinogenesis and in the primary and secondary gastric cancers.

Alternative splicing (AS) is a process by which the exons of primary transcripts (pre-mRNA) from genes can be spliced in different arrangements to produce structurally and, in most cases, functionally distinct mRNA and protein variants [10]. Several splicing variants of *S100A4* gene have been reported, of which two isoforms with Exons 1, 3, and 4 (AS1) and with Exons 2, 3, and 4 (AS2) were well documented [11,12]. According to the Gene Database [www.ncbi.nlm.nih.gov/entrez], the translation starting point of *S100A4* mRNA was located at the Codon 1013 in Exon 3 rather than in Exon 1 or 2, resulting in the same protein when AS1 or AS2 was used as translation templates. For this reason, more efforts were paid to investigate the oncogenic effects of S100A4 protein; consequently, the genetic implication and prognostic value(s) of these two splicing variants in human cancers had long been overlooked.

So far, several molecular alterations have been proposed to be responsible for S100A4 up-regulation. E-cadherin is an important adhesion molecule that distributes widely in epithelial cells. This protein is regarded as an invasion suppressor, because loss of functional membranous E-cadherin results in epithelial-mesenchymal transition, a precedent condition of cancer dissemination [13]. An inverse relationship of S100A4 and E-cadherin expressions was found in some types of cancers including gastric cancer [14], which was closely related with the poorer outcome of cancer patients. However, it is still unclear whether these two alterations are directly linked or indirectly paralleled. Recent data obtained from cultured colorectal cancer cells suggested that S100A4 was the target of Wnt signaling because S100A4 promoter contains a T-cell factor (TCF) binding site that could be activated by β-catenin, providing a cue for Wnt-oriented antimetastasis approach [15]. Nevertheless, similar *in vivo* findings have not yet been available. DNA methylation is a common epigenetic event for regulating gene transcription [16]. The data from other cancer types reveal frequent hypomethylation of CpG sites in the first intron of *S100A4* gene [17], but the casual relationship of this event with gene reactivation has not yet been well ascertained and no datum has been so far available to correlate S100A4 up-regulation with hypomethylation in gastric cancers.

On the basis of the above unclear issues, the current study aimed to profile expression incidence and splicing pattern(s) of S100A4 during stepwise gastrocarcinogenesis and to elucidate the underlying reason(s) that account for S100A4 expression using a panel of gastric samples in different morphologies and from different sources.

Materials and Methods

Sample Collection and Treatment

Sixty-five gastric cancer cases were selected from the Frozen Gastric Tissue Bank in Liaoning Laboratory of Cancer Genomics, Dalian Medical University (DMU). They were obtained from the operating rooms of DMU-Affiliated Hospitals after getting patients' consents. The tissues were chosen and incised carefully from the tumor mass, tumor-surrounding tissue, and grossly normal-looking epithelium, respectively. They were trimmed into suitable sizes on ice, snap-frozen immediately in liquid nitrogen, and stored at −80°C until use. All treatments were done within 20 minutes after removal. Regional lymph nodes were obtained during operation and fixed in 10% formaldehyde for pathological diagnosis and then for experimental purposes. After getting patients' consent, fresh endoscopic gastric biopsies of 18 GC-free patients were collected from the Gastroendoscopic Department of DMU Second Affiliated Hospital. The samples were initially used for *Helicobacter pylori* test and then for research purposes. The tonsil specimens removed by conventional tonsillectomy were treated in the same manner and used as S100A4-positive controls [6] in immunohistochemical (IHC) staining, reverse transcription–polymerase chain reaction (RT-PCR), and Western blot analysis. After getting donor's consent, the culture of normal fibroblasts was conducted using a piece of normal skin specimen removed for cosmetological purpose. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). The cells were harvested and used as S100A4-positive control in immunocytochemical (ICC), protein, and RNA evaluations.

Hematoxylin and eosin staining and morphologic reexamination were performed on each of the frozen tissue blocks before further experiments. When the blocks showed homogeneous composition, they were sectioned directly for RNA and protein isolations followed by frozen tissue array construction. Otherwise, the target histologic region(s) were defined and sampled for cell type–defined sample preparations and tissue array construction by the methods described elsewhere [18]. RNA isolation was performed on the tiny endoscopic biopsies without cell discrimination.

Cell Culture and Treatment

Human gastric cancer cell lines MGC803 and BGC823 were cultured in DMEM (Gibco, Los Angeles, CA) containing 10% FBS at 37°C in 5% CO_2. After getting donor's consent, the culture of normal fibroblasts was conducted using a piece of normal skin specimen removed for medical cosmetic purpose. The cells were incubated only or cocultured with GC cells in DMEM containing 10% FBS and were used as S100A4-positive control. The cells were harvested for DNA, RNA, and protein preparations when closing to 80% of confluence. For ICC and immunofluorescence examinations, gelatin-coated coverslips were put onto the dishes before initial cell seeding. The cells were harvested for ICC and immunofluorescence staining, RNA isolation, and protein extractions when they reached a semiconfluent state.

Tissue Array–Based Immunohistochemical Staining

The representative donor tissues were sampled by coring needles under −30°C and embedded orderly into a frozen recipient block prepared from fresh chicken muscle (Chinese Invention Patent: 02109826.3) to create frozen tissue microarrays. The frozen tissue microarrays with densities of 36 or 56 spots/cm^2 were sectioned to 5-μm thickness for IHC profiling. The antibodies used were as follows: a rabbit anti–human polyclonal antibody that specifically recognized S100A4 out of other members in S100 family (Lab Vision & Neomarkers, Fremont, CA; 1:100), a mouse anti–human–E-cadherin monoclonal antibody (NCH-38; DAKO, North America Inc. CA; 1:120), a goat anti–human Wnt2 polyclonal antibody (Santa Cruz Biotech, Santa Cruz, CA; 1:100), a rabbit anti–human–TCF4 polyclonal antibody (Santa Cruz Biotech; 1:120), and a mouse anti–-human–β-catenin monoclonal antibody (Santa Cruz Biotech; 1:100). The microarray sections lacking incubation with individual primary

antibodies were used as background controls. The staining results were evaluated by two researchers, with the density of immunolabeling scored as negative (–) if no immunolabeling was observed in target cells, weakly positive (+) if the labeling was faint, moderately (++) to highly positive (+++) when the labeling was distinctly strong than (+), or focally positive (F+ ~ +++) if only part of target cells showed + and/or >++ staining patterns.

RNA Extraction and RT-PCR

Sample RNA were isolated from defined histologic regions of frozen tissue blocks, using TRIzol Reagent (Life Tech, Grand Island, NY). Reverse transcription–polymerase chain reaction was performed using Takara RNA PCR kit (AMV) version 2.1 (Takara Biotech Inc, Dalian, China) under the following conditions: 0.8 μg of total RNA was reverse-transcribed with random primer at 55°C for 30 minutes in a 20-μl solution; the reaction was terminated by incubating the mixture at 99°C for 5 minutes. S100A4 was amplified using a pair of primers: sense, 5′-TCA GAA CTA AAG GAG CTG CTG ACC -3′; antisense, 5′-TTT CTT CCT GGG CTG CTT ATC TGG -3′. Polymerase chain reaction was performed with 30 cycles at 94°C for 4 minutes, 94°C for 45 seconds, 59°C for 45 seconds, and 72°C for 1 minute, followed by final extension at 72°C for 7 minutes [19]. The splicing variants of S100A4 (Figure 1) were amplified by two pairs of primers (sense AS1, 5′-CTC TCT ACA ACC CTC TCT CC-3′; sense AS2, 5′-GCA CAC GCT GTT GCT ATA GTA C-3′, antisense AS1/AS2, 5′-GGA AGA CAC AGT ACT CTT GG-3′) under the condition with 35 cycles at 95°C for 4 minutes, 55°C for 45 seconds, and 72°C for 1.5 minutes, followed by 72°C for 7 minutes for the final extension. β-Actin products generated from the same RT solution were referred as internal quantity controls. To clearly demonstrate 9 base pair (bp) difference between AS1 (287 bp) and AS2 (278 bp), the two kinds of PCR products were separated in 6% polyacrylamide gels (containing 6 ml of H_2O, 2 ml of 5 × Tris-borate-EDTA solution, 2 ml of 30% acrylamide, 70 μl of 10% ammonium persulfate, and 6 μl of 4*N*-tetramethylethylenediamine) and photographed with the automatic UV illuminator (Bio-spectrum Imaging System; UVP, Inc., Upland, CA).

Protein Preparations and Western Blot Analyses

Eight to sixteen pieces of 5-μm dissected fragments from defined frozen tissue regions were collected for protein preparations. They were put into 1.5-ml Eppendorf tube containing 150 μl of prechilled 2 × Laemmli SDS sample buffer containing 0.125 M Tris-HCl pH 6.8, 4% SDS, 20% v/v glycerol, and 0.2 M dithiothreitol. After being homogenized by ultrasonic vibration (UP200s; Hielscher GmbH, Mannheim, Germany), the samples were heated in boiling water for 5 minutes. Cell lysates were centrifuged for 4 to 5 minutes at 15,000 rpm, and the protein-containing supernatants were removed to fresh tubes for experimental purposes.

For Western blot analysis, the 50-μg protein samples were separated in 10% SDS-PAGE gel and then transferred to polyvinylidene difluoride membrane (Amersham Inc., Buckinghamshire, UK). The membrane was blocked with 5% skim milk (Becton Dickinson France SA, Le Pont-de-Claix, France) in TBS at 4°C overnight, incubated at room temperature for 1 hour with the first antibodies recognizing human S100A4 (Santa Cruz Biotech) and for another hour with horseradish peroxidase–conjugated goat antimouse IgG. The bound antibody was detected using the enhanced chemiluminescence (ECL) system (Roche GmbH, Mannheim, Germany). After removing the labeling signal by incubating with stripping buffer (62.5 mM Tris-HCl, pH 6.7, 100 mM 2-mercaptoethanol, 2% SDS) at 55°C for 30 minutes, the membrane was reprobed with E-cadherin and then with β-actin using the same experimental procedures.

Identification of the Source of S100A4 Variants

To identify the source of S100A4 variants in the premalignant and noncancerous mucosa, RNA samples were isolated from the total mucosa, stromal components (mainly lymphocytes), and relative pure epithelial cells of three representative chronic gastritis that were S100A4-positive in RT-PCR and Western analyses but showed variable S100A4 staining patterns among different cell components of the tissue sections. Briefly, the target stromal and epithelial regions in hematoxylin and eosin–stained slides were determined under the microscope and its margin was marked in a series of tiny spots inked with a special instrument named Lencil (Chinese Inventive Patent: 02109826.3), a lens-like device fixed on the light microscope with a spray tip that could mark the center of the visual field exactly when upward pressure was formed by gently attaching the slide with the spray tip. Referring to the marks on the section, the border of target region in the frozen tissue block was incised with a rotation miniknife (Chinese Inventive Patent: 02109826.3) in the depth of 0.1 to 0.2 mm. By this way, the target fragment separated from the main part during frozen section. According to the size of target region, 6 to 10 pieces of 7-μm frozen fragments were collected immediately by an autoclaved tip wetted with cold TRIsol (Gibco) for RNA isolation. The sample RNA were amplified with the primers for AS1 and AS2 transcripts and separated in 6% polyacrylamide gel. The RNA samples isolated from human tonsil and cultured human fibroblasts were used as controls of stroma-associated S100A4 variant(s).

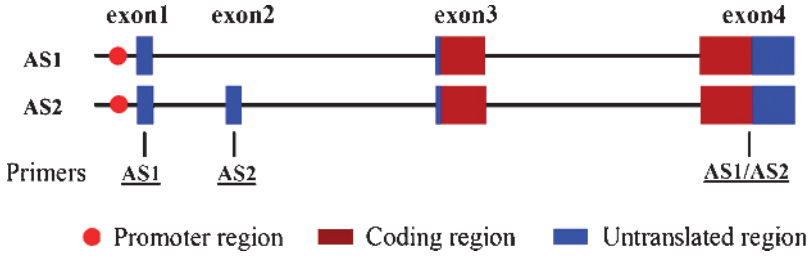

Figure 1. Layout of the composition of splicing variants AS1 and AS2, the site of S100A4 promoter and the locations of PCR primers for AS1 and AS2 amplification.

Correlative Analyses of S100A4 Expression, E-cadherin Reduction, and Wnt Activation

To evaluate the correlation of S100A4 with E-cadherin alterations and Wnt activation, 27 gastric cancer samples (15 i-GC and 12 d-GC) were selected from the 65 cases for a parallel study on the two parameters by the methods of IHC staining. The Kruskal-Wallis and Mann-Whitney tests were used to analyze the relation of S100A4 and E-cadherin expression in different histologic groups. The Spearman correlation test was used to determine the relationship between S100A4 expression and the extent of membranous E-cadherin reduction. TCF4 and β-catenin nuclear cotranslocation is the end point of Wnt signaling [20]. For this reason, this parameter was adopted for evaluating potential association of S100A4 expression and activated Wnt signaling by checking intracellular distribution of TCF4, β-catenin, and S100A4, respectively. The two established GC cell lines, MGC803 and BGC823, were examined by S100A4 ICC staining combined with immunofluorescent double labeling for TCF4 and β-catenin. The cultured normal fibroblasts were used as positive control for S100A4.

DNA Extraction and Methylation Sequencing

Altogether, 7 S100A4-negative gastric mucosa with mild chronic inflammatory changes, 6 S100A4-negative GC tissues, 12 S100A4-positive GC tissues, and 2 GC cell lines were selected for this assay. DNA preparations were conducted in parallel with RNA isolation by the method described above. Sixteen to 24 pieces of 3-μm target frozen fragments were collected after sectioning and placed immediately in a 1.5-ml Eppendorf tube containing 300 μl of Tris-EDTA buffer (pH 8.0, 1% SDS), digested with 5 μl of proteinase K (10 mg/ml), and subjected to conventional DNA extraction using phenol/chloroform extraction and ethanol precipitation. For chemical DNA modification, 5 μl of sample DNA (0.4 μg/μl) was mixed with 40 μl of pure water, heated at 37°C for 10 minutes, and interacted with 5 μl of 2 M NaOH at 37°C for 10 minutes, mixed with 30 μl of freshly prepared 10 mM hydroquinone (H9003; Sigma Chemical Inc., St. Louis, MO) and 520 μl of 3 M sodium bisulfate (S9000; Sigma Chemical Inc.), and then incubated under 50°C in darkness for 16 to 20 hours. The chemical-modulated sample DNA was subjected to desalt purification using Wizard DNA Clean-Up System (A7280; Promega Cooperation, Madison, WI). The purified DNA was dissolved with 50 μl of pure water, mixed with 5.5 μl of 3 M NaOH at 37°C for 5 minutes, precipitated in ethanol, and dissolved in 30 μl of pure water for use [31,32].

A pair of primers as follows was used for sequencing critical CpG sites within the first intron of the *S100A4* gene, according to the Gene Database [www.ncbi.nlm.nih.gov/entrez]: S100A4-F, 5′-GTT TTT TAG GTG TTT TTG AGA TGT G -3′; and S100A4-R, 5′-ACA AAA ACC CAC AAT TAC CTT CTA C-3′. The region amplified by the primers included four CG sites because the methylation status of this site is related with the S100A4 expression [21]. The modified DNA samples were amplified using Takara PCR amplification Kit (Takara Biotech Inc.), and the products were sequenced in reverse direction by Takara Biotech Inc. Dalian Branch with ABI PRISM 3730XL DNA Analyzer and ABI PRISM 377XL DNA sequencer.

Statistical Analyses

Kruskal-Wallis and Mann-Whitney tests were used to analyze the differences of S100A4 detection and the incidences of AS1 and AS2 variants between 1) noncancerous mucosa and premalignant lesions or GC tissues, 2) premalignant lesions and GC tissues, 4) intestinal metaplasia and chronic gastritis, and 5) d-GCs and i-GCs and to elucidate the relation of S100A4 with E-cadherin reduction and Wnt activation in different histologic groups. The Spearman correlation test was used to determine the relationship between S100A4 expression and the extent of membranous E-cadherin reduction as well as TCF4 and β-catenin nuclear cotranslocation.

Results

Frequent Expression and Heterogeneous Distribution of S100A4 in GCs

Histologic reevaluation was performed carefully on each of the tissue blocks before further studies. Totally, 12 noncancerous mucosa, 39 premalignant (24 chronic gastritis and 15 intestinal metaplasia), 65 cancer tissues, and 26 lymph node metastases were checked immunohistochemically. According to Lauren's classification [22], the cancer tissues were classified into two subtypes: relatively well-differentiated intestinal gastric cancer (i-GC) and poorly differentiated diffuse gastric cancer (d-GC). Of 65 GC tissues, 36 were found with distinct heterogeneous composition and therefore subjected to tissue-defined RNA and protein isolations to minimize cell-cell cross-contamination [18]. The corresponding regions were then used for frozen tissue array construction. The sample preparations were also performed on the cultured fibroblasts and human tonsil specimens.

The results of array-based IHC staining were summarized in Table 1 and demonstrated in Figure 2*A*. It was found that the epithelial cells in noncancerous tissues were negative in S100A4 expression, but the stromal lymphocytes were stained positively; the detection rate of S100A4 increased to 13% (5/39) in premalignant tissues and reached to 75% (49/65) in cancer samples, especially those metastasized to lymph nodes (26/26, 100%). Statistical analyses with Kruskal-Wallis and Mann-Whitney tests revealed that S100A4 detection rate of tumor tissues was significantly different with that of noncancerous mucosa and premalignant lesions ($P = .000$) but neither between noncancerous and premalignant mucosa ($P = .196$) nor between i-GC and d-GC ($P = .057$). The amount and distribution of S100A4 protein were not identical among the primary cancer tissues and sometimes in the same glandular structure, because some cells demonstrated enhanced S100A4 immunolabeling, whereas others showed much

Table 1. Tissue Microarray-Based IHC Profiling of S100A4 Expression in Different Gastric Tissues.

Histologic diagnosis	*n*	S100A4 (%)			*P*
		–	+/>++	Focal +~+++	
Noncancerous	12	12 (100)	0/0 (0/0)	0	
Premalignant	39	34 (87)	4/1 (10/3)	0	.196*
Chronic gastritis	24	22 (92)	1/1 (4/4)	0	
Intestinal metaplasia	15	12 (80)	3/0 (20/0)	0	.295‡
Gastric cancer	65	16 (25)	14/17 (21/26)	18 (28)	.000*,†
i-GC	45	8 (18)	12/11 (27/24)	14 (31)	
d-GC	20	8 (40)	2/6 (10/30)	4 (20)	.057§
Lymph node metastases	26	0 (0)	26 (100)		
i-GC	16	0 (0)	16 (100)		
d-GC	10	0 (0)	10 (100)		

*In comparison with noncancerous mucosa.
†In comparison with premalignant lesions.
‡In comparison with chronic gastritis.
§In comparison with i-GC.

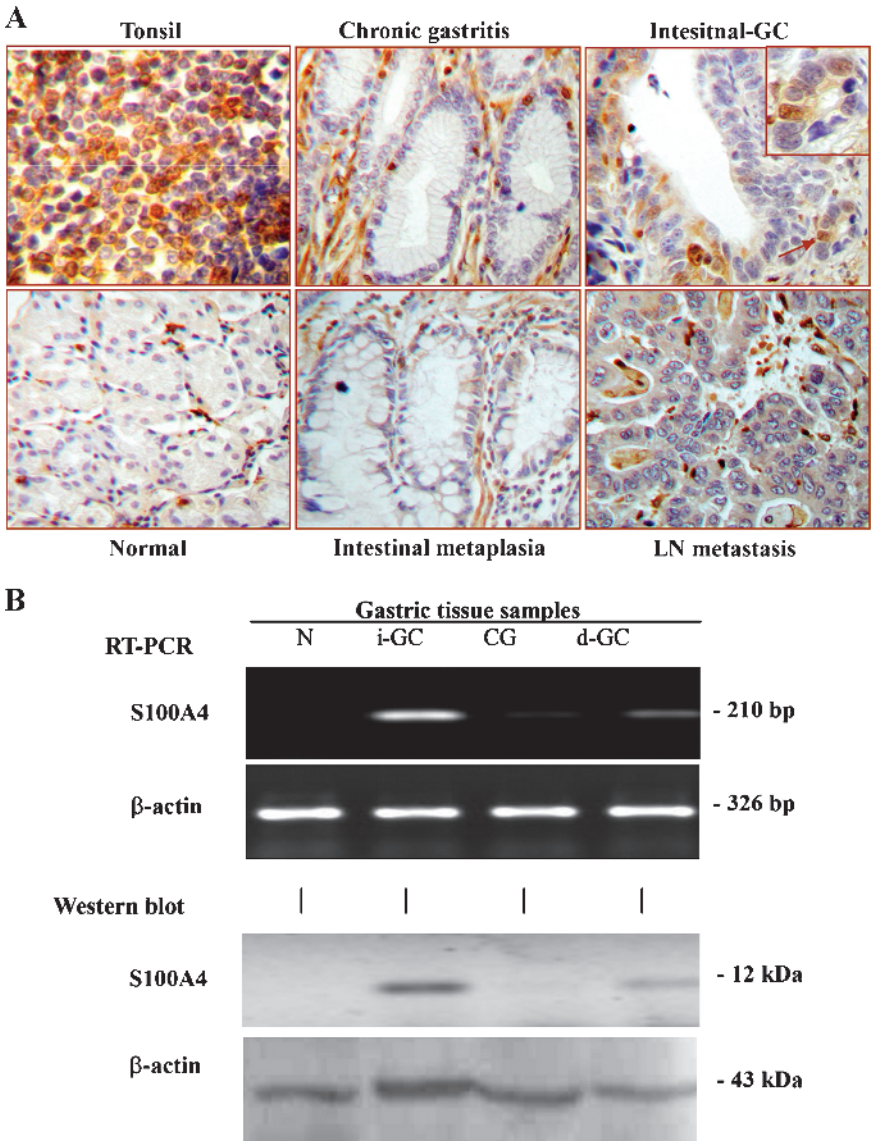

Figure 2. (A) Immunohistochemical illustration (original magnification, ×40) of S100A4 expression in noncancerous mucosum (N), chronic gastritis (CG), intestinal metaplasia (IM), and a case of intestinal type gastric cancer (i-GC) with lymph node metastasis (LN+). Human tonsil tissue was used as S100A4-positive control. Arrow indicates the gland shown in higher magnification (original magnification, ×80). (B) Evaluation of S100A4 expression in some gastric tissues by RT-PCR and Western blot analyses. β-Actin was used as quantitative and qualitative control.

weaker, even negative, staining (Figure 2*A*; i-GC). This focal positive phenomenon was found in 18 of 65 GCs so far checked (Table 1). Conversely, all of the lymph node metastases were stained homogenously irrespective of the different S100A4 distribution in their primary tumors (Figure 2*A*; lymph node and Table 1).

Preferential Expression of S100A4 AS2 Variant in GC Cells

To validate the IHC data, Western blot and RT-PCR analyses were performed on the protein and RNA samples isolated from 7 noncancerous, 19 premalignant, and 26 cancer regions of 26 GC cases. As shown in Figure 2*B*, S100A4 protein in 12 kDa could be observed in i-GC and d-GC samples but was undetectable or was very weak in their noncancerous or premalignant counterparts. The two major forms of S100A4 splicing variants, AS1 and AS2, were amplified from the same RT products under identical reaction condition. As indicated in Table 2 and shown in Figure 3*A*, the detection rates

Table 2. Reverse Transcription–Polymerase Chain Reaction Detection of S100A4 Splicing Variants (AS1 and AS2) in Different Gastric Tissues.

Histologic diagnosis	*n*	AS1 (%)		*P*	AS2 (%)		*P*	*P*
		−	+		−	+		
Noncancerous	7	6 (86)	1 (14)		7 (100)	0 (0)		.620*
Premalignant	19	3 (16)	16 (84)	.030†	14 (74)	5 (26)	.406†	.002*
Gastric cancer	26	2 (8)	24 (92)	.009†	9 (35)	17 (65)	.019†	.019*
				.399‡			.010†	
d-GC	12	0 (0)	12 (100)		3 (27)	8 (73)		.069*
i-GC	15	2 (13)	13 (87)	.574§	6 (40)	9 (60)	.610‡	.104*
GC-free biopsies¶	18	0 (0)	18 (100)		15 (87)	3 (13)		.000*
GC cell lines	2	0 (0)	2 (100)		0 (0)	2 (100)		

*In comparison with AS1.
†In comparison with noncancerous mucosa.
‡In comparison with premalignant lesions.
§In comparison with i-GC.
¶RNAs were isolated without cell discrimination.

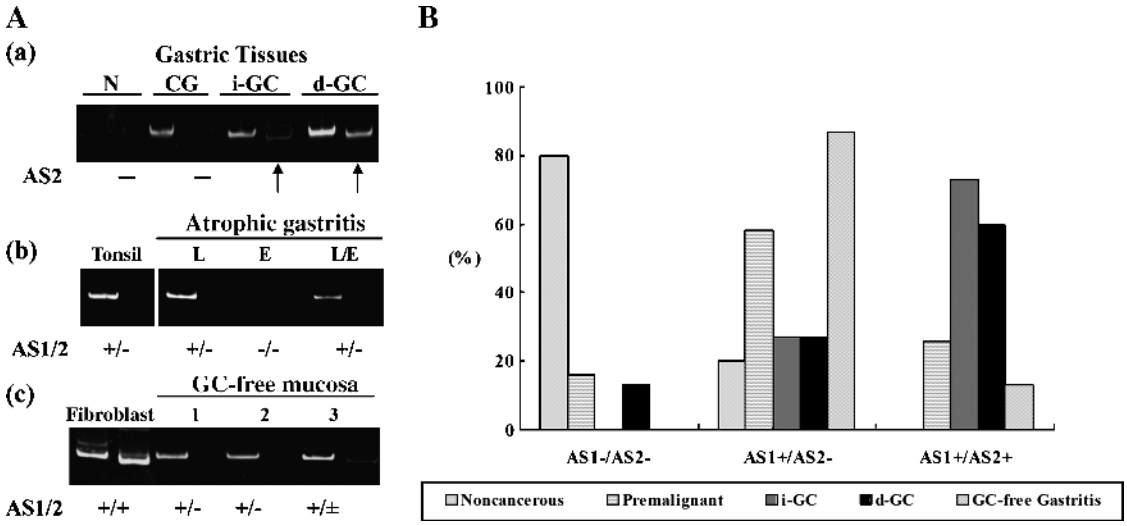

Figure 3. (A) The numbers and percentages of AS1−/AS2−, AS1+/AS2−, and AS1+/AS2+ cases in noncancerous epithelia, premalignant lesions, intestinal gastric cancer (i-GC), diffuse gastric cancer (d-GC) groups, and among the endoscopic gastric mucosa obtained from GC-free patient. AS1−/AS2+ splicing pattern is not found in the samples so far checked. (B) Characterization of AS patterns of S100A4 in noncancerous, premalignant, and cancer tissues of the stomach (a), the main components of chronic gastritis tissue obtained from GC patient (b) and endoscopic mucosa of GC-free patients (c) by RT-PCR and polyacrylamide gel electrophoresis. Human tonsil and cultured normal fibroblasts were used as S100A4-positive controls. Arrows indicate the samples with AS2 expression.

of AS1 transcript were 14% (1/7) among noncancerous mucosa, 86% (16/19) among premalignant lesions, and 92% (24/26) among cancer tissues including 100% (11/11) of i-GC and 87% (13/15) of the d-GC samples. The frequencies of AS1 expression were significantly different between noncancerous and premalignant (P = .030) or GC tissues (P = .009) but not between premalignant and GC tissues (P = .399). AS2 was undetectable in noncancerous epithelia (0/7) but became detectable in 26% (5/19) of premalignant tissues and in 66.6% (18/27) of GCs. The frequencies of AS2 detection were significantly different either between noncancerous and GC tissues (P = .019) or between premalignant and GC samples (P = .010). The differences of AS2 detection between noncancerous and premalignant tissues and between i-GCs and d-GCs were not statistically significant (P = .406 and P = .610, respectively). Additionally, the levels of AS1 and AS2 expression vary with the samples except in cultured fibroblasts (Figure 3*B*).

S100A4 Splicing Patterns Vary with Cell Types

RNA samples were isolated from human tonsil, the total specimens, and the defined regions of stromal lymphoid nodules and epithelial layers of some chronic gastritis and cultured normal fibroblasts, respectively. By the use of those RNA samples, the sources of AS1 and AS2 variants in premalignant lesions and S100A4 splicing patterns in different cell types were characterized by RT-PCR and PAGE assay. As shown in Figure 3, *A* and *B*, only the AS1 product could be detected in the sample RNA isolated from the tonsil and the lymphoid nodules (L) of chronic gastritis. The AS1 product was also detectable from the RNA samples isolated from the total premalignant specimen (L/E) but not from the epithelial cells (E). However, both AS1 and AS2 products were generated in almost equal amounts from the cultured fibroblasts (Figure 3*A*, *a–c*). Among the tissue samples so far checked, no case was found with only the AS2 expression (AS1−/AS2+).

Rarity of AS2 in Inflammatory Mucosa of GC-Free Patients

RNA samples were prepared from gastric mucosa of 18 GC-free patients without cell discrimination. Reverse transcription–polymerase chain reaction analyses for AS1 and AS2 revealed that AS1 transcript could be detected in those samples (Figure 3*A*, *a–c*, and Table 2), whereas a very faint AS2 band was observed only in a few of samples

Table 3. Level of S100A4 Expression and Abundance of Membranous E-cadherin and Nuclear β-Catenin in Different Gastric Tissue.

Histologic Diagnosis	*n*	S100A4				*P*	E-cadherin			*P*	Nuclear β-Catenin				*P*
		−	+	++	+++		−	+	>++		−	+	++	+++	
Noncancerous	7	7	0	0	0		0	0	7		7	0	0	0	
Premalignant	27	26	0	1	0		5	9	13		21	4	1	1	
Chronic gastritis	16	15	0	1	0		3	4	9		13	2	0	1	
Intestinal metaplasia	11	11	0	0	0	.801*	2	5	4	.527*	8	2	1	0	.501*
Gastric cancer	27	6	10	8	3	.000†	10	14	3	.007†	1	13	8	5	.000†
i-GC	15	2	5	6	2		4	10	1		1	6	5	3	
d-GC	12	4	5	2	1	.152‡	6	4	2	.131‡	0	7	3	2	.755‡

*In comparison with chronic gastritis.
†In comparison with premalignant lesions.
‡In comparison with i-GC.

Table 4. Correlations of S100A4 with Membranous E-cadherin and Nuclear β-Catenin Expression in GCs.

		S100A4				*r*	*P*
		–	+	++	+++		
E-cadherin	–	7	5	3	0	−0.282	.043
	+	9	4	5	4		
	>++	14	1	2	0		
β-Catenin	–	18	0	2	0	0.313	.024
	+	6	5	3	3		
	++	4	2	4	0		
	+++	3	4	0	0		

(3/18). In contrast, sufficient amounts of AS1 and AS2 products were generated from the sample RNA of normal fibroblasts. Similar with the findings in noncancerous mucosa of GC patients, IHC staining demonstrated that the lymphocytes and sporadically distributed fibroblasts in the stromal space but not epithelial cells were positive in S100A4 expression (data not shown).

Inverse S100A4 and E-cadherin Expressions Are Not Always Overlapped

In parallel with the S100A4 examination, the status of membranous E-cadherin expression in different gastric tissues was evaluated immunohistochemically. As shown in Table 3, membranous E-cadherin remained intact (++~+++) in all noncancerous (7/7) and in most premalignant lesions (13/20, 52%) but became decreased (+) and even diminished (–) in 89% (24/27) of GCs. The difference of membranous distribution of E-cadherin is significant between the two groups (P = .007). Although correlative analyses of S100A4 and E-cadherin expressions (Table 4) revealed a general inverse relationship among the cancer and premalignant samples so far checked (P = .043, r = −0.282), there were two cases of GCs with S100A4 up-regulation and almost unchanged level of E-cadherin expression and, conversely, 7 of 54 cases (2 GC and 5 premalignant tissues) with neither E-cadherin nor S100A4 expression. As demonstrated in Figure 4*A*, E-cadherin was generally distributed in glandular crypts of an i-GC case but part of cells showed S100A4 expression. Furthermore, both MGC803 and BGC823 cells expressed S100A4, whereas the former showed almost intact membranous E-cadherin (Figure 4*B*).

S100A4 Expression and Wnt Activation Were Not Well Matched

Nuclear cotranslocation of β-catenin and TCF4 has been regarded as the consequence of Wnt activation [27,30,35]. Of 54 gastric samples compared here, some premalignant lesions and majority of gastric cancers were found with this cotranslocation (Table 1 and Figure 5*A*). Meanwhile, S100A4 was found in 21 of 27 GC samples but only in 1 premalignant mucosum. Although a general correlation of these two parameters could be established statistically (P = .039, r = 0.313; Table 2), there were 13 (38%) of 34 cases with β-catenin/TCF4 nuclear translocation but absence in S100A4 expression and 2 (8.7%) of 23 S100A4-positive GC cases without β-catenin/TCF4 nuclear translocation (Figure 5*A*). The *in vitro* study demonstrated that both S100A4 expression and β-catenin/TCF4 nuclear translocation were found in MGC803 and BGC823 cells, whereas the cultured human fibroblasts expressed S100A4 in the absence of β-catenin/TCF4 nuclear translocation (Figure 5*B*).

S100A4 Hypomethylation in GC Tissues and Cell Lines

The *S100A4* gene does not contain CpG islands in the promoter region, but methylation of critical CpG sites in the first intron regulated transcription of the *S100A4* gene [39,21]. To determine the

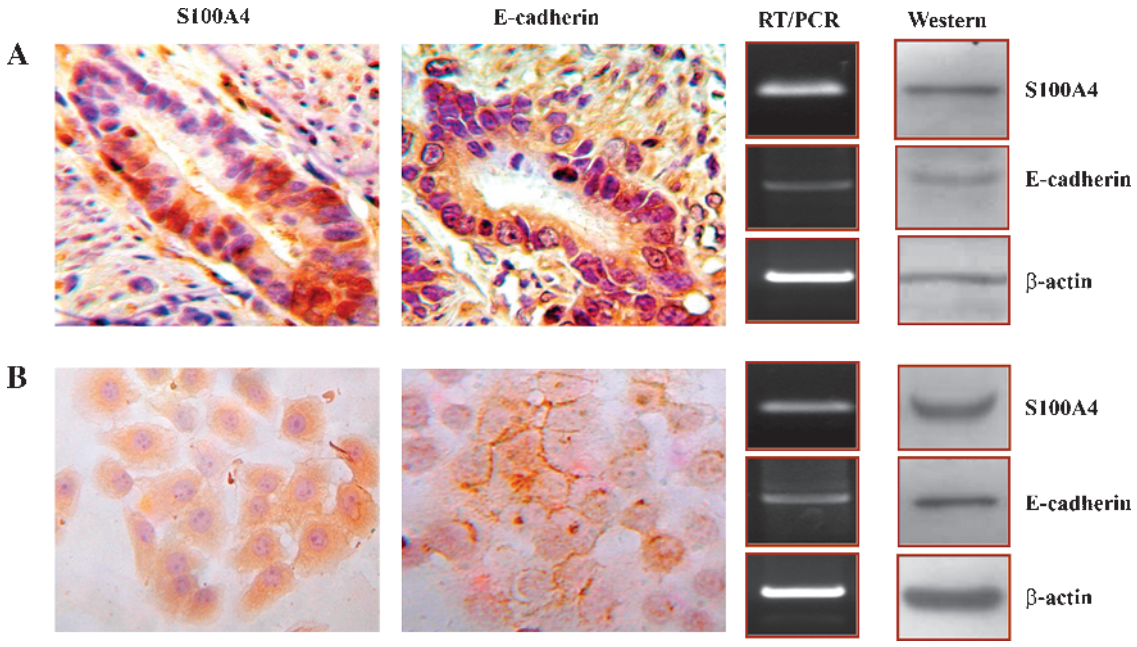

Figure 4. Paralleled analyses of S100A4 and E-cadherin expressions in a glandular structure of i-GC tissues (A) and BGC823 cell line (B) by IHC staining, RT-PCR, and Western blot analysis. Heterogeneous S100A4 expression and uniformly distributed E-cadherin could be observed in (A), and coexistence of up-regulated S100A4 and membranous E-cadherin in (B). Original magnifications were ×25 for tissues and ×40 for cultured cells.

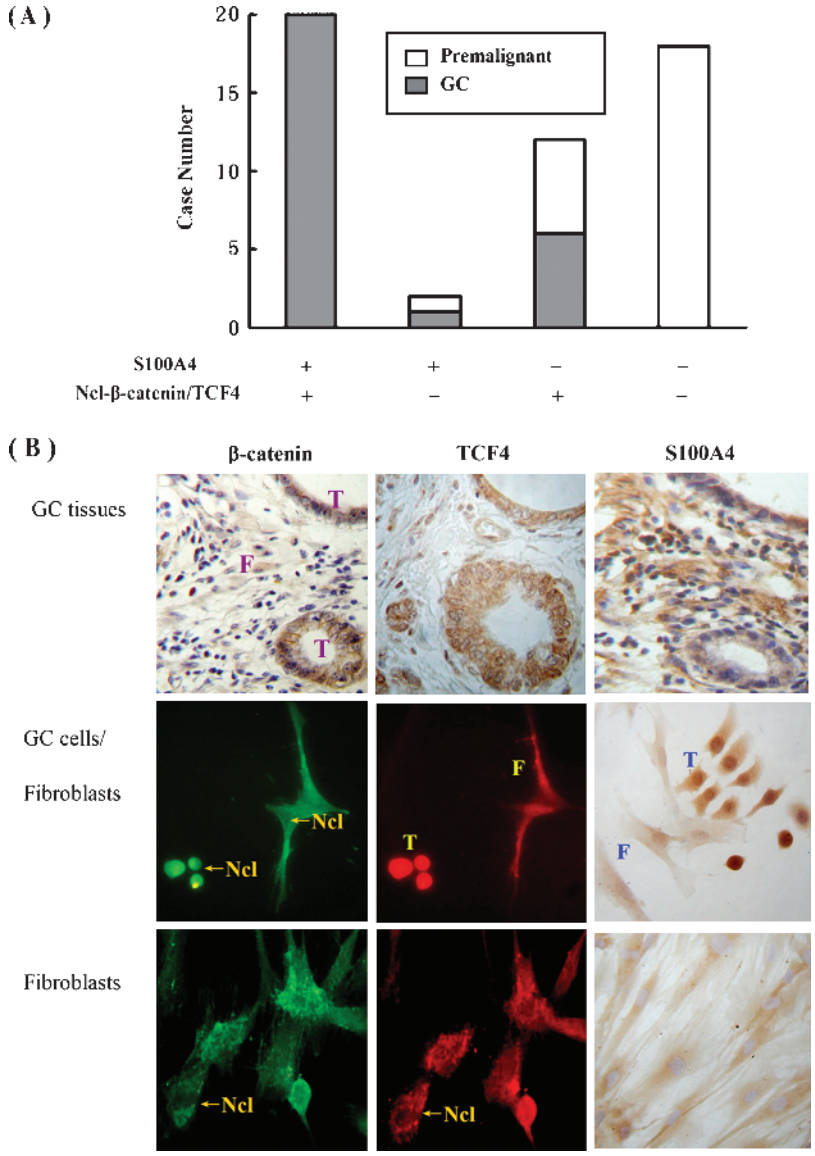

Figure 5. (A) Fractionation of 52 gastric tissues (27 GCs and 25 premalignant lesions) used in this study according to their statuses of S100A4 expression and β-catenin/TCF4 nuclear cotranslocation. (B) Immunohistochemical illustration of β-catenin, TCF4, and S100A4 expression in cancer cells and their surrounding stromal fibroblasts of an intestinal gastric cancer case. S100A4 ICC staining and double-labeled immunofluorescent illustration of β-catenin (green) and TCF4 (red) performed on the coculture of human GC cell line (BGC823) and normal fibroblasts and on the cultured normal fibroblasts. *F* indicates cultured fibroblasts; *Ncl*, nucleus; *T*, GC cells. Original magnification, ×80.

relation of DNA methylation status with S100A4 expression in GC tissues and cell lines, PCR amplification and product sequencing were performed on sodium bisulfite–modified DNA obtained from 7 noncancerous gastric mucosa, 6 S100A4-negative GC tissues (2 i-GCs and 4 d-GCs), 12 S100A4-positive (7 i-GCs and 5 d-GCs), and 2 GC cell lines. As shown in Table 5, all of the four CpG sites in the first intron were methylated in the sample DNA of noncancerous gastric mucosa and S100A4-negative GC tissues. In contrast, the guanine/G molecules in the four CpG sites were replaced by adenine/A in the DNA isolated from the S100A4-expressing GC tissues and GC cell lines. A close correlation of S100A4 expression and CpG site methylation was statistically established (P = .000). Figure 6 demonstrates the methylation status of the third and the fourth CpG sites in the first intron of noncancerous mucosum, cancer tissue, and BGC823 cell lines. The same methylation patterns were found in the first and the second CpG sites but not shown in Figure 6 because of the space limitation.

Discussion

Although the association of overexpressed S100A4 with metastasis potential of gastric cancers has been described [23], the detailed S100A4 expression pattern(s) during stepwise gastrocarcinogenesis remains less known. Our IHC results are in line with the general notion of the close link of S100A4 expression and GC dissemination [9,23] by showing frequent S100A4 detection in primary GCs (75%) and their lymph node metastases (100%). Further analysis showed that the gastric epithelia of 18 GC-free patients were stained

Table 5. Methylation Statuses of the Four CpG Sites in S100A4 First Intron Among the Gastric Tissues With (+) and Without (–) S100A4 Expression.

Group	Case No.	S100A4*	
		Hypermethylation	Hypomethylation
S100A4–			
Noncancerous†	7	7	0
GCs	6	6	0
S100A4+			
GC tissues	12	0	12
GC cell lines	2	0	2

*Methylational modulations concurrently happen to the four CpG sites in the first intron.
†Gastric mucosa with mild chronic gastritis.

negatively in S100A4, suggesting the later appearance of S100A4 expression in the course of gastrocarcinogenesis. It has been known that S100A4 can be expressed by the components of tumor stromal components such as tumor-associated fibroblasts, lymphocytes, and endothelial cells [24–26]. The case also holds true in premalignant and cancer tissues of the stomach because of the frequent observation of S100A4-positive cells in stromal components and tumor surrounding tissues. For instance, S100A4 was expressed in the fibroblasts and lymphocytes of premalignant lesions such as atrophic gastritis, presumably owing to the intrinsic [27] and/or inflammatory reason(s) as observed in renal epithelia with chronic injuries [28]. The above findings thus implicate 1) that S100A4-mediated parenchyma-stroma interplays may occur as early as premalignant stage, 2) that S100A4-producing stromal cells may play favorable roles in cancer promotion and progression as suggested by Emberley et al. [29], and 3) that the contamination of S100A4-positive stromal cells during sample preparation may elevate the rates of S100A4 detection in Western blot analysis and, especially, RT-PCR examination.

The status of S100A4 expression has been studied on many kinds of cancers, but the detailed distribution patterns of this protein in cancer tissues have rarely mentioned till recently. Agerbaek et al. [30] reported the focal S100A4 expression in primary bladder cancers and its predictive value in patients' prognosis. According to our observation, d-GCs usually exhibit homogenous distribution of *S100A4* protein, whereas *S100A4* is distributed heterogeneously in i-GCs. Interestingly, this protein is allocated uniformly in all of lymph node metastases irrespective of its distribution patterns in the corresponding primary tumors. The above phenomena implicate that *S100A4* may confer on GC cells' more aggressive behavior and selective advantage for successful distant metastasis by the biological effects proposed by many investigators [24,31,32]. The underlying reason(s) leading to the heterogeneous *S100A4* distribution remains obscure at current stage, but it is unlikely caused by the so-called multiclonal GC formation because of the heterogeneous S100A4 expression in the same cell clusters or glandular structures of primary tumors. Alternatively, the highly variable feature of S100A4 expression might indicate the influence(s) of microenvironmental element(s), cell cycle regulators, and especially epigenetic factor(s) in the transcription of this gene [33].

Alternative splicing is a universal posttranscriptional event, which provides the basis of proteomic generation and functional diversity

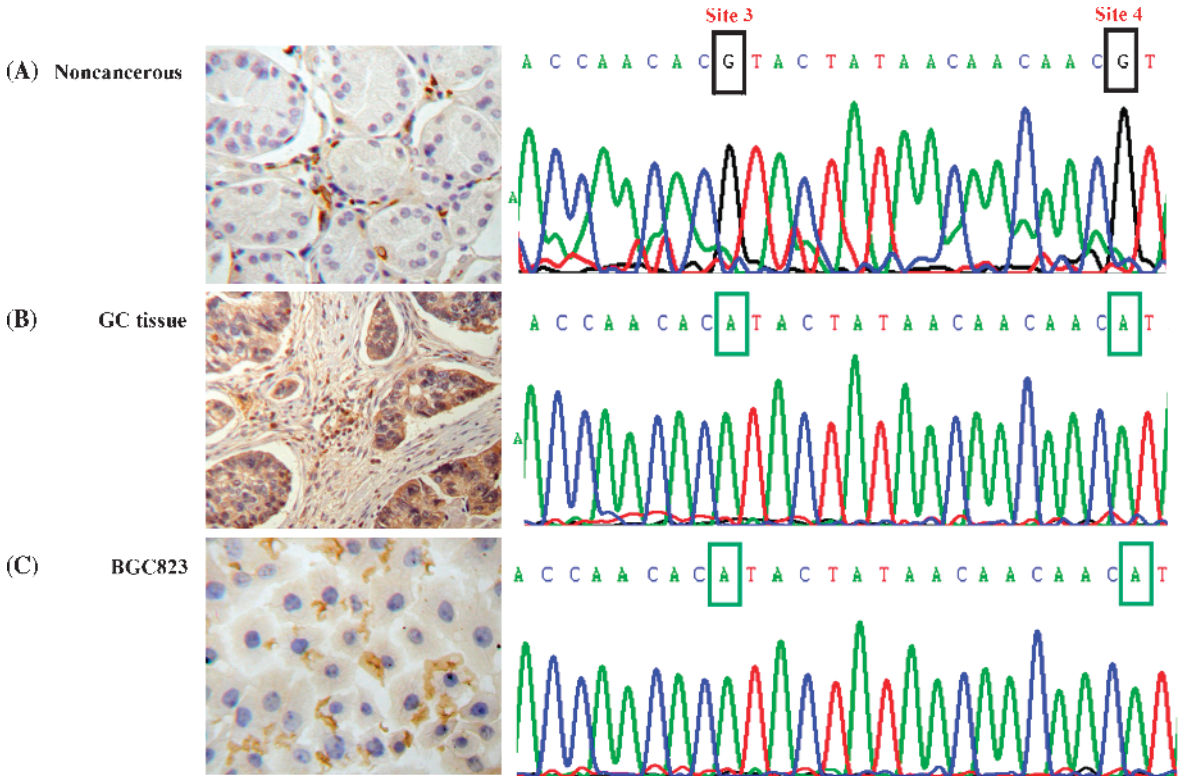

Figure 6. Representative PCR-generated sequences (in complementary pairing and reverse direction) of the S100A4 intron 1 from sodium bisulfate–treated DNA samples of S100A4-negative noncancerous mucosum (A), S100A4-positive GC tissue (B), and BGC803 cell line (C) showing the third and fourth sites of CpG methylation in (A) for base C protected and hypomethylated in (B) and (C) for base C transfer to T.

of cells [10]. The ways of splicing are usually different between normal tissues and their malignant counterparts [34]. Alternative splicing also happens to the *S100A4* gene, and several splicing variants with the sequence changes ahead of promoter region have been described [11,12]. However, no data have been so far available about their biological and oncological implications. It has been recognized that the ASs that occurred in the noncoding sequences of the gene transcripts may not change the protein sequence but sometimes reflect cellular alterations [34]. Therefore, the expression patterns of two relatively stable S100A4 splicing variants [11] in different gastric tissues were examined by checking the migration patterns of RT-PCR products in polyacrylamide gel. In additional to the high frequency of S100A4 detection in GC samples, we found a 287-bp PCR product harboring Exons 1, 3, and 4 (AS1 variant) in premalignant lesions of some GC cases as well as the endoscopic chronic gastritis of GC-free patients without stroma discrimination and an additional 9-bp shorter product encompassing Exons 2, 3, and 4 (AS2 variant) in a great part of GCs. These findings strongly suggest that AS2 is a unique splicing isoform generated preferentially in GC cells and is therefore closely associated with malignant transformation of gastric epithelial cells. It is interesting that AS1 transcript is constantly expressed in S100A4-positive cells irrespective of the cell types, whereas none of the tissue samples so far checked has been found with a splicing pattern of AS1-negative and AS2-positive (–/+). Given the evidence of the absence of AS1 and AS2 (AS1–/AS2–) in normal gastric mucosa, frequent AS1 but not AS2 (AS1+/AS2–) in premalignant lesions, and increased AS1 and AS2 detection (AS1+/AS2+) in GC tissues, it would be possible that AS1 may be a highly conserved transcript or a standard form of S100A4, whereas AS2 comes out as a later event. To test this speculation, the origin(s) of AS1 and AS2 transcripts in premalignant lesions of GC patients was clarified by RT-PCR performed on the individual cell components. The AS1 variant was generated from the lymphoid nodules and from the tonsil lymphocytes but not from the epithelial cells of premalignant lesions and GC surrounding tissues. To ascertain these phenomena, IHC staining was performed on endoscopic mucosa, which showed immunolabeling only in the stromal lymphocytes and sporadically appeared fibroblasts. These evidences prove largely 1) that normal gastric epithelial cells rarely express S100A4 and 2) that S100A4-expressing stromal but not epithelial cells are responsible for the increased RT-PCR detections of S100A4 in premalignant tissues. Therefore, the data obtained from *in situ* IHC observation may provide more reliable information. Conversely, both AS1 and AS2 variants were detected in the cultured fibroblasts, suggesting that GC cells share the capacity with fibroblasts to mutually exclude Exon 2 or Exon 1 during the process of splicing. Although it is difficult to predict the biological significance of GC- or fibroblast-associated AS2 expression at the current stage, the high frequency of this variant in tumor samples indicates, at least in part, that S100A4 AS2 may be one of the representative global splicing abnormalities of gastric cancers. The absence of AS1–/AS2+ splicing pattern may be caused by the apparent existence of exon 1 on both splice variants and implicates that the appearance of AS2 is the secondary event of S100A4 expression. This "exonomic" change may serve as a potential diagnostic and prognostic biomarker of gastric cancer.

So far, the detailed molecular mechanism leading to disordered S100A4 expression remains obscure. Several hypotheses have been proposed to explain the onset of this unfavorable molecular event, which include the potential inhibitory effect of E-cadherin on S100A4 expression [34], the targeting transcriptional activation of S100A4 by Wnt signaling [15], and the epigenetic modulation of S100A4 expression through DNA methylation at the CpG sites in the first intron of the *S100A4* gene [12]. However, no paralleled correlative study has been done to evaluate the influence of these three elements in S100A4 expression. This issue was addressed in current study using identical experimental gastric system.

E-cadherin has been known as metastasis-inhibitory protein by forming intercellular junction [15]. Reduced E-cadherin expression has been found in a variety of malignancies including GCs owing to genetic and/or epigenetic reasons [13,14]. Inverse expression patterns of S100A4 and E-cadherin are commonly found in different cancers, but the casual relationship between these two alterations has not yet been ascertained. Although our statistical analysis showed this inverse correlation as well, the gain of S100A4 and loss of E-cadherin did not always occur concurrently in gastric cancers. For instance, the coexistence of S100A4 expression and intact membranous distribution of E-cadherin was found in 15% (4/27) primary GCs. In some cases, E-cadherin is distributed evenly in the glandular structures of i-GCs but part of cells showed S100A4 expression. Besides, down-regulated E-cadherin could be observed in five S100A4-negative GC cases. These data thus implicate the alternative regulation of S100A4 expression in gastric cancer cells.

Nuclear translocation of β-catenin, another unfavorable molecular event that happened during the later stages of gastrocarcinogenesis, is considered as the consequence of inverse expressions of Wnt and E-cadherin [35,36]. Recently, S100A4 was proposed as a target of Wnt signaling because a β-catenin/TCF–binding site had been identified in the S100A4 promoter sequence of cultured colon cancer cells [15]. However, *in vivo* correlative analysis of β-catenin/TCF nuclear translocation and S100A4 expression has not yet been available. Our comparative analysis clearly revealed that these two alterations were overlapped in 17 of 27 GCs, suggesting their importance in GC formation and progression. We also noticed that S100A4 expression was not necessarily accompanied with β-catenin/TCF nuclear translocation, because Wnt activation was absent in S100A4-positive premalignant lesions as well as the stromal fibroblasts. The same situation could also be found in the cultured human fibroblasts in which S100A4 was constitutively expressed irrespective of the absence of Wnt expression and β-catenin/TCF nuclear translocation. These findings thus indicate the existence of additional regulatory machinery for S100A4 expression in premalignant gastric lesions and fibroblasts.

DNA methylation is the main machinery of epigenetic modulation, which regulates the gain or the loss of gene transcription. Hypermethylation of tumor-suppressor genes and hypomethylation of cancer-associated genes are common epigenetic phenomena in cancer cells. In most types of normal cells, the *S100A4* gene keeps silence and becomes expressed when malignant transformation occurs to those cells [3,37]. The data from prostate cancer, colon cancer, and pancreatic cancer revealed that S100A4 expression was closely related with hypomethylation of CpG sites in its first intron [15,38,39]. Given the evidence of S100A4 expression beyond E-cadherin down-regulation and Wnt activation, the methylation status in that region was checked using a panel of S100A4-negative and -expressing gastric samples. It was clearly demonstrated that all of S100A4-expressing GC tissues/cell lines were found with the hypomethylation of the four intronic CpG sites, irrespective of the morphologic subtypes; in contrast, those sites remain hypermethylated in the noncancerous gastric mucosa and the six S100A4-negative GC tissues. Our current findings

thus highlight the role of hypomethylation in regulating S100A4 expression in gastric cancers, although the consensus of intronic scattered CpG site methylation with gene expression needs to be further confirmed by the use of DNA demethylators and/or methylators.

Collectively, this study demonstrated for the first time the distinct S100A4 distribution patterns between some primary GCs and their lymph node metastases, the differential S100A4 splicing patterns among individual cell elements of gastric mucosa, the preferential S100A4 AS2 expression in GC tissues and human fibroblasts, and the absence of AS1−/AS2+ splicing pattern in different samples so far checked. The above findings suggest that S100A4 expression and, especially, the appearance of AS2 variant would have potential values in the assessment of GC risk and prognosis. S100A4 expression is correlated but not always overlapped with E-cadherin downregulation and Wnt activation in gastric cancers. Nevertheless, the status of CpG site methylation in the first intron was closely related with the silence or the gain of S100A4 transcription, suggesting the importance of this epigenetic factor in modulating S100A4 expression in gastric cancers. As the next step, the biological implication of fibroblastlike splicing patterns of S100A4 in GC cells and the effect of DNA demethylator on S100A4 expression will be investigated.

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